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Article

Immobilization of cellulase onto MnO₂ nanoparticles for bioethanol production by enhanced hydrolysis of agricultural waste

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ABSTRACT

Cellulase is an efficient enzymatic catalyst that hydrolyses cellulosic substances. The high costs associated with using enzymes for industrial applications can be reduced by immobilizing the cellulase. In the current study, cellulase produced by *Aspergillus fumigatus* JCF was immobilized onto MnO₂ nanoparticles, which improve the activity of cellulase and offer a superior support. The surface characteristics of synthesized MnO₂ nanoparticles and cellulase-bound MnO₂ nanoparticles were investigated by scanning electron microscopy, and Fourier transform infrared spectroscopy was used to analyze the functional characteristics of the immobilized cellulase. The maximum cellulase binding efficiency was 75%. The properties of the immobilized cellulase, including activity, operational pH, temperature, thermal stability, and reusability were investigated and were found to be more stable than for the free enzyme. It was found that cellulase immobilized on MnO₂ nanoparticles could be used to hydrolyze cellulosic substances over a broad range of temperature and pH. The results confirmed that cellulase immobilized on MnO₂ nanoparticles was very efficient in terms of cellulolytic activity.

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1. Introduction

Cellulose is a major biopolymer found in plants that has many industrial applications if efficiently used, but a major portion of it is wasted in forest and agricultural waste. The key step in exploiting cellulose is its hydrolysis into monomeric sugars and their eventual conversion into valuable compounds for the release of energy [1]. Among the various methods available for cellulose hydrolysis, enzymatic methods are the most efficient. Cellulase refers to a group of enzymes that include endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β -glucosidases (EC 3.2.1.21) [2,3]. It plays an important role in the conversion of cellulose into monomeric units of glucose, which can be subsequently used for the production of various biochemicals [4].

The use of cellulase for enzymatic degradation of cellulose requires large enzyme loadings. This increases the production cost and economic demands [5]. There are many methods for improving the efficiency of enzymatic activity. Immobilization of cellulase will increase its enzymatic stability and enable its reuse, which could reduce the cost of cellulose degradation. Thus, immobilization of the enzyme will increase the overall productivity of the monomeric units resulting from the degradation reaction. Various materials, such as chitin [6], chitosan [7], nylon [6], and polyvinyl alcohol (PVA) [8], have been used as supports for immobilized cellulase.

The economics of enzyme-based hydrolysis of biomass can be improved by increasing the thermal stability, efficiency, and reusability of enzymes. Improvement of all of these properties can be achieved by the immobilization of enzymes on support

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matrixes [9]. This system can also be used for the production of bioethanol, which is of great importance in the current era. The simultaneous saccharification and fermentation (SSF) process, first described by Annamalai et al. [10], combines enzymatic hydrolysis of cellulose with simultaneous fermentation of its main derived sugar (glucose) to ethanol. Consumption of limited available fuel resources is increasing at an enormous rate, which is a major threat globally, but problems in ensuring energy security can be overcome by using alternative energy sources [11,12]. Bioethanol produced through saccharification and fermentation is a promising solution to this situation [13], and fuels produced by this method are considered to be environmentally friendly.

Nanoparticles have been used extensively in many applications such as biomedicine [14], targeted drug delivery [15], biosensors [16], water purification [17], protein immobilization [18], and environmental remediation [19]. Nanoparticles can also be used for the immobilization of enzymes. The use of nanostructured materials for the immobilization of enzymes may increase biocatalytic efficiency through the increased enzyme loading arising from their large surface areas [20].

Many metal oxides are currently used for the immobilization of cellulase [21]. Amongst the various metals used, Mn significantly improves cellulase activity [22]. MnO₂ nanoparticles have been synthesized using several methods, including hydrothermal [23], combustion [24], and co-precipitation methods [25]. Two commonly used techniques for immobilizing enzymes onto nanoparticles are covalent binding and physical adsorption. Covalent binding is achieved by the formation of covalent bonds between the enzyme and the nanoparticle [26] and is considered the most reliable way to minimize protein desorption.

In the current study we focused on the synthesis of nanoparticles by a co-precipitation method. The MnO₂ particles were characterized by scanning electron microscopy (SEM). The immobilization of cellulase onto MnO₂ particles was validated by Fourier transform infrared spectroscopy (FT-IR). The properties of the immobilized cellulase, including thermal, pH, and operational stability were also investigated. The current study also focused on SSF of agricultural waste using the immobilized enzyme for the production of bioethanol.

2. Experimental

2.1. Production of cellulase enzyme by submerged fermentation

Cellulase enzyme was produced from a previously isolated *Aspergillus fumigatus* JCF using jackfruit waste as a substrate. The jackfruit waste and sugar cane leaves were brought to the lab and dried overnight at 60 °C and mill-ground. The substrate taken from 240-μm mesh was pretreated with NaOH and used as the carbon source. Pretreatment of the jackfruit reduces cellulose crystallinity by removing lignin and hemicelluloses, and NaOH was selected as the pretreatment chemical based on our previous work. The substrate was added to modified Mandel's medium, sterilized, inoculated with *Aspergillus fumigatus* JCF and maintained in an orbital shaker for 4 d at 120 r/min to

produce cellulase. Conditions for the production of cellulase had previously been optimized and the maximum cellulase activity produced was about 3.3 IU/mL.

2.2. Synthesis of MnO₂ nanoparticles

A chemical co-precipitation method was used to synthesize MnO₂ nanoparticles. A 1 mol/L MnSO₄·H₂O solution was dissolved in deionized water, and 2 mol/L of NaOH was then added dropwise. The solution was stirred continuously at 60 °C for 2 h to precipitate the nanoparticles. Precipitated particles were then collected and washed with deionized water 2–3 times and dried in a hot air oven at 100 °C for 12 h [27].

2.3. Immobilization of cellulase onto MnO₂ nanoparticles

The MnO₂ nanoparticles were suspended in deionized water at a concentration of 5 mg/mL. This suspension was sonicated for 1 h, after which it was added to a glutaraldehyde solution (1 mol/L) in deionized water [28]. Support activation was achieved by incubating the MnO₂ nanoparticles for 1 h at 25 °C in a shaker at 250 r/min. The activated magnetic support was washed twice with deionized water.

The covalent binding of the enzyme to nanoparticles was achieved by incubating the activated nanoparticle support with enzyme at a concentration of 5 mg/mL at 25 °C for 2 h in a shaker at 250 r/min. The supernatant obtained after separating the immobilized particles from solution was used for protein estimation. The immobilized enzyme on the nanoparticles was subsequently thoroughly washed with deionized water and buffer to remove weakly bound cellulase [29].

2.4. Enzyme binding efficiency

The enzyme binding efficiency was found by calculating the ratio of protein bound to nanoparticles (C_b) to the total protein available for binding (C_t). Protein concentration was determined by the Bradford assay [30]. The amount of bound enzymes was calculated as $(C_i - C_s)$, where C_i and C_s are the concentrations of the enzyme initially added for attachment and the residual in the supernatant, respectively (mg/mL).

$$\text{Binding efficiency (\%)} = (C_b/C_i) \times 100$$

In the current study cellulase was attached to the nanoparticles with the help of glutaraldehyde. Glutaraldehyde is considered as a good binding agent because it helps to immobilize the enzyme onto nanoparticles without disturbing the active site of the enzyme. Enzyme immobilization was carried out for 2 h, and the maximum binding efficiency in current study was found to be 75%. This indicates that 75% of the total protein added was immobilized effectively on MnO₂ nanoparticles.

2.5. Characterization of MnO₂ nanoparticles with and without enzyme

The morphology and size of the nanoparticles before and after immobilization of enzyme were studied with the aid of a field-emission SEM from CARL ZEISS, Germany and energy

dispersive spectroscopy (EDS) from OXFORD Instruments, United Kingdom. Synthesized nanoparticles were characterized using an FT-IR instrument from 13-ELMER, USA. Characterization of the functional groups attached to the surface of the synthesized MnO_2 nanoparticles was carried out with a scanning range of $4000\text{--}400\text{ cm}^{-1}$ and a resolution of 4 cm^{-1} .

2.6. Kinetics of free and immobilized enzyme

The Michaelis–Menten kinetics constant (K_m) was calculated by measuring the reaction rate of free and immobilized enzyme using different concentrations of carboxymethyl cellulose (CMC) as substrate in a citrate buffer at $50\text{ }^\circ\text{C}$. The concentration of CMC was varied from 5 to 50 mg/L. The K_m and $V_{\max}$ (maximum reaction velocity) values of free and immobilized enzyme were calculated using a Lineweaver–Burk plot [31]. The effect of temperature on the activity of free and immobilized enzyme was studied by varying the temperature from 30 to $80\text{ }^\circ\text{C}$. The pH was varied from 4 to 8. The thermostability of free and immobilized enzyme was determined at optimum temperature. The cellulase enzyme assay was carried out at intervals of 1 h. Immobilized enzymes usually display increased stability and reusability. To study the reusability of cellulase immobilized on MnO_2 nanoparticles, after determining the enzymatic activity, nanoparticles immobilized with cellulase were recovered and washed with demineralized water. These were then inoculated into freshly prepared substrate for further assay, with the cellulase activity obtained in the first assay taken as the control (100% activity).

2.7. Bioethanol production by SSF

In the process of SSF, lignocellulosic materials with complex cellulose are broken down into simple sugars by cellulolytic action and simultaneously fermented to produce bioethanol. The yeast *Saccharomyces* sp. was selected to produce bioethanol because it achieves a higher bioethanol production efficiency than other strains [32]. One gram of pretreated sugar cane leaves was mixed with 100 mL citrate buffer in a 250-mL flask. Media was prepared by mixing yeast extract (0.1%, w/v) and peptone (0.1%, w/v) into the above solution. The media was then sterilized and inoculated with baker's yeast and 4 mL

of free or immobilized enzyme. The fermentations were carried out at 120 r/min and $30\text{ }^\circ\text{C}$. Samples were collected at regular intervals of 12 h. Residual sugar and ethanol concentrations were determined for each sample.

2.8. Assay of cellulase enzyme activity

The reducing sugars concentration was determined by the dinitrosalicylic acid (DNS) method [33]. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1.5 mL of 1% CMC in 50 mmol/L sodium citrate acetate buffer (pH 4.8) and 1.5 mL of filtrate. The reaction mixture was incubated at $50 \pm 2\text{ }^\circ\text{C}$ for 10 min, and the reducing sugars produced were determined as follows. To the reaction mixture, 3 mL of DNS reagent was added and incubated for 15 min at $100\text{ }^\circ\text{C}$. The reaction mixture was cooled, 1 mL Rochelle salt was added, and the optical density was taken at 575 nm. One unit (IU) of cellulase activity was defined as the amount of enzyme releasing $1\text{ }\mu\text{mol}$ of reducing sugars per min.

2.9. Determination of bioethanol

The amount of ethanol produced in the fermentation media was estimated using the dichromate method. Cell-free extract (1 mL) was diluted four times and 1 mL of potassium dichromate was added, while maintaining all tubes containing the mixture in ice water. Concentrated sulfuric acid (5 mL) was added gently through the walls and then the optical density was measured on a spectrophotometer at 660 nm [34].

3. Results and discussion

3.1. Characterization of MnO_2 nanoparticles with and without cellulase enzyme

The morphology of MnO_2 nanoparticles was studied by SEM. SEM images of the nanoparticles before and after the binding of cellulase are shown in Fig. 1(a) and (b), respectively. The average size of nanoparticles was 76 nm before immobilization of cellulase (Fig. 1(a)) and 101 nm after the immobilization (Fig. 1(b)). An EDX analysis was carried out (Fig. 2) to confirm the presence of MnO_2 . It shows a very strong signal for Mn and O,

Fig. 1. SEM images of MnO_2 nanoparticles (a) and cellulase immobilized on MnO_2 nanoparticles (b).

Fig. 2. EDX image of MnO_2 nanoparticles.

confirming the existence of MnO_2 .

The binding of cellulase onto the synthesized nanoparticles was confirmed by FT-IR analysis. Figure 3 shows the results obtained from FT-IR, which indicate the purity and nature of the nanoparticles synthesized. Further, the various functional groups associated with nanoparticles were analyzed by FT-IR analysis. The strong peaks at 620 and 505 cm^{-1} clearly indicate the presence of MnO_2 . The absorption peak observed at 3450 cm^{-1} may be attributed to $-\text{CH}_3$ stretching vibrations and NH stretch. The characteristic bands for a protein at 1397, 1290, and 1260 cm^{-1} , respectively, indicate that cellulase was successfully immobilized onto the MnO_2 nanoparticles. The stretch at 1620 cm^{-1} suggests the binding of C=O groups [29,35,36].

3.2. Kinetic studies of free and immobilized enzyme

Using varying concentrations of CMC as a substrate, kinetic constants were evaluated for free and immobilized cellulase. The Michaelis-Menten constant (K_m) and the maximum reaction velocity ($V_{\max}$) of free and immobilized enzyme were calculated from a Lineweaver-Burk plot. The K_m values of free and immobilized enzyme were found to be 6.43 and 3.56 mg/mL , respectively. The immobilized enzyme showed a slight increase in the K_m value compared with the free enzyme. This indicates that the immobilized enzyme had a higher affinity towards the substrate than free enzyme, which may have been caused by

the immobilization and subsequent conformational change of the enzyme. The $V_{\max}$ was found to change from 0.66 $\text{mg mL}^{-1} \text{ min}^{-1}$ for the immobilized enzyme to 0.86 $\text{mg mL}^{-1} \text{ min}^{-1}$ for the free enzyme. The catalytic efficiency of the reaction system increased from 0.102 min^{-1} for free enzyme to 0.24 min^{-1} for immobilized enzyme. A similar result was observed in a study conducted on the immobilization of cellulase on TiO_2 [37].

3.3. Enzyme activity at various initial pH values and reaction temperatures

The optimum initial pH for the activity of an enzyme is mainly dependent upon the nature of its functional groups. However, coupling of the enzyme to a support can shift the pH range of its activity [38,39]. As shown in Fig. 4(a), the maximum activity of immobilized enzyme occurred at pH = 5, compared with pH = 6 for the free enzyme. This may have been caused by an increase in net charge arising from the binding of the enzyme to the MnO_2 nanoparticle.

The effect of temperature on the activities of both free and immobilized cellulase was studied in the range of 40–80 $^{\circ}\text{C}$ because the activity of cellulase is highly dependent upon temperature. The enzyme activity can be observed to increase up to a temperature of approximately 50 $^{\circ}\text{C}$, while it shifts to a higher range around 70 $^{\circ}\text{C}$ for the immobilized cellulase (Fig. 4(b)). The increase in optimum temperature range may have been caused by reduced conformational flexibility, which requires a higher activation energy for the molecule to reorganize its proper conformation to bind to the substrate [40], thus activity even at higher temperature is one of the main advantages of enzyme immobilization.

3.4. Thermostability and reusability

The thermostability results of free and immobilized cellulase enzyme are shown in Fig. 4(c). Immobilized enzyme was found to be more stable than free enzyme. The activity of enzyme immobilized on MnO_2 nanoparticles was found to be stable for 2 h but the activity of the free enzyme was found to decrease after 1 h. Immobilized enzyme retained 89% of its original activity after 2 h of reaction time. After 2 h enzymatic activity was observed to decrease gradually, while the stability of

Fig. 3. FT-IR analysis of MnO_2 nanoparticles (1) and cellulase immobilized on MnO_2 nanoparticles (2).

Fig. 4. Effect of pH (a) and temperature (b) on the activities, and the thermostability (c) and reusability (d) of free (1) and immobilized cellulase (2).

free enzyme was found to decrease sharply. Thus, cellulase immobilized on MnO_2 nanoparticles was inactivated at a much slower rate than the free enzyme.

One of the main advantages of enzyme immobilization is the reusability of enzyme, which will reduce the cost of the enzyme. The immobilized enzyme was used several times to hydrolyze cellulose. After each use, the nanoparticles were washed thoroughly with deionized water. It was observed that even after the fifth cycle, the enzyme maintained around 60% of its original activity (Fig. 4(d)). This reduction in enzyme activity may have been caused by several mechanisms, including protein denaturation, loss of one or more components from the cellulase complex, or structural modification of enzyme [39]. Cellulase immobilized on magnetoresponsive graphene showed an enzymatic activity of 55% after four cycles of use [32].

3.5. Bioethanol production by SSF

Plant biomass generally contains 40%–50% cellulose, 20%–40% hemicelluloses, and 20%–30% lignin by dry weight [41,42]. Therefore, in this study, cheaply available agricultural waste and sugar cane leaves were used to produce reducing sugars and thereby bioethanol. Powdered sugar cane leaves were pretreated with 0.5 mol/L NaOH prior to SSF to remove lignin and hemicelluloses. The resultant biomass was used as the substrate for bioethanol production. In addition to the pretreated sugar leaves, peptone and yeast extract were added, and the medium was sterilized. The sterilized medium was inoculated with immobilized enzyme and yeast in one flask and free enzyme and yeast in a second flask.

Fig. 5. Reducing sugar concentration and bioethanol production in SSF catalyzed by free (1) and immobilized cellulase (2).

Powdered sugar cane leaves were treated with free or immobilized cellulase enzyme, which released reducing sugars that were acted upon simultaneously by yeast to produce bioethanol. The highest amounts of reducing sugars released from sugar leaves when treated with free and immobilized enzyme were about 20 and 24.29 g/L, respectively (Fig. 5(a)). The maximum amounts of bioethanol produced by SSF from sugar cane leaves were about 18 and 21.96 g/L, respectively, using free and immobilized enzyme (Fig. 5(b)). Thus, immobilization of cellulase improved the enzymatic activity and thereby increased bioethanol production.

4. Conclusions

The binding of cellulase onto MnO_2 nanoparticles was successfully carried out and confirmed by FT-IR spectroscopic analysis. The characteristic size of the nanoparticles by SEM (76 nm) was found to increase to 101 nm after the immobilization of cellulase. The immobilized cellulase on nanoparticles were found to be more thermostable than free enzyme, and the immobilized enzyme was stable at an optimum temperature of 70 °C for 2 h. Reusability of cellulase was also greatly increased after immobilization. Immobilized enzyme retained 60% activity even after five cycles. Cellulase immobilized on MnO_2 nanoparticles, in combination with yeast for SSF, yielded a bioethanol concentration of about 21.96 g/L.

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Graphical Abstract

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Immobilization of cellulase onto MnO_2 nanoparticles for bioethanol production by enhanced hydrolysis of agricultural waste

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This study presents the synthesis of MnO_2 nanoparticles and immobilization of cellulase on the nanoparticles, and demonstrates their application in bioethanol production by simultaneous saccharification and fermentation (SSF).

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MnO₂纳米粒子固载纤维素酶用于高效水解农业废弃物制备生物乙醇

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摘要: 纤维素酶是一种有效的纤维质类物质水解催化剂, 工业应用时可通过固定化纤维素酶来降低其成本. 本文将烟曲霉原变种 JCF产生的纤维素酶固定在MnO₂纳米颗粒上. MnO₂可提高纤维素酶的活性, 并充当一个更好的载体. 采用扫描电镜表征了所制 MnO₂纳米粒子及其负载纤维素酶的表面性质, 以傅里叶变换红外光谱分析了固定在MnO₂纳米粒子上纤维素酶的官能团性质. 纤维素酶在MnO₂纳米粒子上最大的固定化效率为75%. 考察了固定化纤维素酶的活性、操作pH值、温度、热稳定性和重复使用性等性质. 结果表明, 所制固定化酶的稳定性比游离酶更高. 固定于MnO₂纳米粒子上的纤维素酶可用于纤维质类物质的水解反应, 且能在较宽的温度和pH值范围内使用. 表征结果证实了该催化剂具有非常高的催化纤维素类物质水解的活性.

关键词: 纤维素酶; 固定化; 二氧化锰; 纳米生物催化剂; 农业废弃物; 水解; 生物乙醇

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